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Subfunctionalization and neofunctionalization of vertebrate Lef/Tcf transcription factors

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ABSTRACT

Invertebrates express a multitude of Wnt ligands and all Wnt/β-catenin signaling pathways converge to only one nuclear Lef/Tcf. In vertebrates, however, four distinct Lef/Tcfs, i.e. Tcf-1, Lef, Tcf-3, and Tcf-4 fulfill this function. At present, it is largely unknown to what extent the various Lef/Tcfs are functionally similar or diversified in vertebrates. In particular, it is not known which domains are responsible for the Tcf subtype specific functions. We investigated the conserved and non-conserved functions of the various Tcfs by using Xenopus laevis as a model organism and testing Tcfs from Hydra magnipapillata, Caenorhabditis elegans and Drosophila melanogaster. In order to identify domains relevant for the individual properties we created series of chimeric constructs consisting of parts of XTcf-3, XTcf-1 and HyTcf. Rescue experiments in *Xenopus* morphants revealed that the three invertebrate Tcfs tested compensated the loss of distinct Xenopus Tcfs: Drosophila Tcf (Pangolin) can substitute for the loss of XTcf-1, XTcf-3 and XTcf-4. By comparison, Caenorhabditis Tcf (Pop-1) and Hydra Tcf (HyTcf) can substitute for the loss of only XTcf-3 and XTcf-4, respectively. The domain, which is responsible for subtype specific functions is the regulatory CRD domain. A phylogenetic analysis separates Tcf-1/Lef-1 from the sister group Tcf-3/4 in the vertebrate lineage. We propose that the vertebrate specific diversification of Tcfs in vertebrates resulted in subfunctionalization of a Tcf that already united most of the Lef/Tcf functions.

Introduction

The Wnt/Wg signaling cascade is one of the most conserved signaling cascades in the animal kingdom. It is essential for the early patterning of invertebrate and vertebrate embryos (Fuerer et al., 2008, Nusse, 2005). For cnidarians it is even discussed that a Wnt code precedes the function of the Hox-code in patterning higher animals (Guder et al., 2006).

An almost complete set of Wnt gene subfamilies known from deuterostomes is expressed in simple organisms such as the cnidarians *Hydra* (Lengfeld et al., 2009) and *Nematostella* (Kusserow et al., 2005), and expression of multiple Wnts is conserved throughout the animal kingdom, although many protostome clades exhibit significant loss of *Wnt* genes. Many protostomes, including *Drosophila* and Caenorhabditis, express only a handful of Wnt ligands. Sequence alignments allow distinguishing the different Wnt subfamilies and highlighting which particular Wnts got lost in a distinct species.

For the major transcription factors of the Wnt/Wg signaling pathway, the Lymphoid enhancer factor/T cell factors (Lef/Tcfs), the situation is different. While all invertebrates studied so far express only one Tcf, all higher vertebrates produce four different Lef/Tcf family members named Lef-1, Tcf-1, Tcf-3 and Tcf-4 (Hugo names; LEF1, TCF-7, TCF-7 like1 and TCF-7 like2,respectively), which exist in different splice variants (Arce et al., 2006; Hoppler and Kavanagh, 2007, Weise et al., 2010). Knock-out experiments in mice and knockdown studies in *Xenopus laevis* revealed that each of the four family members has its own individual function, which no other Lef/Tcf can take over (Galceran et al., 1999; Gregorieff et al., 2004; Kunz et al., 2004; Liu et al., 2005; Standley et al., 2006; van Venrooy et al., 2008; Koenig et al., 2010). It was also shown that different Lef/Tcfs regulate their target genes differently; Tcf-1 and Lef-1 act as transcriptional activators, while Tcf-3 instead should be considered as a transcriptional repressor. Alternative splicing of Tcf-4 in the context dependent regulatory domain (CRD) and the C-terminus results in isoforms, which act as transcriptional activators or repressors (Weise et al., 2010; Wöhrle et al., 2007; Pukrop et al.,

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2001; Gradl et al., 2002). Thus, Wnt signal transduction by the usage of different transcription factors appears to be a novel achievement of the vertebrate lineage. Thus far it remained elusive whether this diversification of Tcfs in the vertebrate lineage provided distinct family members with novel functions (neofunctionalization), and/or whether existing properties of ancient Tcfs were splitting off and created distinct vertebrate Tcf subfamilies (subfunctionalization).

The overall structure of all Lef/Tcfs is conserved throughout the animal kingdom. They all consist of an N-terminal β-Catenin binding site (βbs), followed by a context dependent regulatory domain (CRD), a high mobility group (HMG) box as DNA binding motif and a C-terminal part.

At a first glance, the sequence homology within the N-terminal *β*-Catenin binding site (βbs) is not extremely high (35% aa identity between HyTcf and XTcf-3); it is always considered to bind to vertebrate β-Catenin. Thus, it seems likely that the conformation of vertebrate Tcf N-termini (Sun and Weis, 2011) is conserved also in invertebrate Tcfs. Indeed, residues including D16 and E24, known to be important for β-Catenin binding to Tcf-4 (Fasolini et al., 2003), are found in all Tcfs (alignment in supplementary Fig. 1).

The context dependent regulatory domain (CRD) which resides between the βbs and the HMG box is even less conserved ($<20\%$ aa identity between HyTcf and XTcf-3). This region is discussed as the modulator domain, which serves as a platform for binding co-repressors of the groucho family (Daniels and Weis, 2005) and other proteins (Valenta et al., 2006), including some specific for a certain family member (Bruhn et al., 1997) or a certain alternatively spliced region (Ghogomu et al., 2006). The competition of *β*-Catenin and groucho relies on an auxiliatory β-Catenin binding domain within the CRD (Daniels and Weis, 2005). The HMG box as a sequence dependent DNA binding motif is the most conserved region in Lef/Tcfs (68.5% identity between HyTcf and XTcf-3). It recognizes a conserved Wnt responsive element (WRE) with the consensus sequence (A/T)(A/T)CAAAG (van de Wetering and Clevers, 1992). Meanwhile it has been shown that distinct family members prefer different sequences (Weise at al. 2010; Atcha et al., 2007). For this modulation, an additional DNA recognition site, the CRARF domain, forms a C-clamp and recognizes an additional GC element downstream of the consensus WRE (Atcha et al., 2007). Interestingly, a similar CRARF domain is present in all invertebrate Tcf C-termini, but only in a subset of vertebrate Tcf isoforms. Alternatively, the C-terminus in vertebrate Tcfs may contain a PLSV/T motif for binding the co-repressor C-terminal binding protein (Ctbp). A similar motif is also found in the two major isoforms of *Drosophila* Tcf (Pangolin), but not in Caenorhabditis Tcf (Pop-1) or in the Tcf of *Hydra* (HyTcf). The current idea is that the specificity of Lef/Tcf functions relies on both, different affinities between distinct WRE, which might differ due to additional DNA recognition by the CRARF domain and recruitment of different co-factors binding to the low conserved CRD and the C-terminus.

With these complex mechanisms regulating the Wnt response in the nucleus in mind, it is surprising that invertebrates like *Hydra* and Caenorhabditis, which express a multitude of distinct Wnt ligands (Kusserow et al., 2005, http://www.stanford.edu/group/nusselab/cgi-bin/wnt/), have only one Tcf to induce different transcriptional responses. While the transcriptional regulation of target genes by Tcfs was only recently investigated (Nakamura et al., 2011), in *Drosophila* it is well known that depending on the context Pangolin acts as a transcriptional activator and a repressor. Even a Pangolin/*β*-Catenin complex can act as a repressing complex (Blauwkamp et al., 2008). Thus, the best studied invertebrate Tcf seems to unite two Lef/Tcf functions, transcriptional activation and repression, which are separated in vertebrates among distinct family members/isoforms. Whether alternative splicing of Pangolin might be a reason for these different functions remains a matter of speculation. In *Caenorhabditis* the switch between activation and repression appears to be regulated by NLK phosphorylation of Pop-1 and subsequently by nuclear retention of the transcription factor. Surprisingly, high levels of nuclear Pop-1 are considered to repress Wnt driven transcription, and low levels, instead allow *Wnt* target gene activation (Phillips and Kimble, 2009).

To clarify the relationship between invertebrate Tcfs and vertebrate Lef/Tcfs, we aimed to study the function of these transcription factors in a phylogenetic context. In gain of function experiments using cell culture and Xenopus embryos, we analyzed whether invertebrate Tcfs can act as activators or repressors. In XTcf-1, XTcf-3 and XTcf-4 morphants we tested which invertebrate Tcf can replace one or more of the Xenopus Tcfs. To our surprise we found that the three invertebrate Tcfs tested compensated the loss of different Xenopus Tcfs in a very specific manner; Pop-1 can replace XTcf-3, HyTcf can replace XTcf-4 and Pangolin can replace XTcf-1, XTcf-3 and XTcf-4. We also identified the domains that are relevant for the Tcf subtype specific functions by performing series of chimeric constructs consisting of XTcf-3 and XTcf-1, and XTcf-1 and HyTcf. Our chimera experiments indicate that for the activation of the siamois promoter and for the induction of ectopic body axes, all four XTcf-1 domains, i.e. βbs, CRD, HMG, and the C-terminal domains are essential. In summary, our data suggest an evolutionary scenario of Tcf subfunctionalization according to which ancient properties of Tcfs were splitting off during vertebrate evolution, generating novel vertebrate Tcf subfamilies with restricted functions.

Results and discussion

Molecular phylogeny of metazoan Tcfs

We performed a phylogenetic analysis using sponge, cnidarian, ecdysozoan, and vertebrate Lef/Tcf sequences. As an out-group we have chosen Tcf from the sponge Amphimedon, which is an out-group to all eumetazoans (Srivastava et al., 2010). Fig. 1A shows a maximum-likelihood tree of Lef/Tcf proteins based on their β-Catenin binding domain, the CRD and HMG box domains, and the C-terminal amino acids. The corresponding alignment is shown as supplementary Fig. 1. Similar to the results of Lin et al. (2006), and a phylogenetic tree computed using MrBayes (supplementary Fig. 2), our data demonstrate that invertebrate Tcfs form an ancient cluster of Tcfs. By comparison, vertebrate Lef/Tcfs can be divided into four distinct subfamilies (Tcf-1, -3, -4, and Lef-1). These vertebrate subfamilies can form two distinct clusters, a Tcf-1/Lef-1 cluster and a Tcf-3/-4 cluster (Fig. 1A). The most likely evolutionary scenario for the origin of these vertebrate Tcfs is that the well documented two whole genome duplications in vertebrates (Force et al., 1999; He and Zhang, 2005) have led to four copies of the ancestral invertebrate Tcf gene. The non-chordate *tcf* genes differ mainly in the length of their CRD and C-terminal domains (Fig. 1B and supplementary Fig. 1). Already the cnidarian Tcfs include a CRARF domain that is comparable to those of ecdysozoan Lef/Tcf proteins as well as to Tcf-1 and Tcf-4 of vertebrates (Hecht and Stemmler, 2003; Lin et al., 2006).

Functional analysis of invertebrate Tcfs in Xenopus

For a functional classification of invertebrate Tcfs in vertebrates, we performed secondary axis assays in Xenopus embryos and reporter gene assays in transiently transfected HEK293 cells. We further tested in Tcf subtype specific assays whether invertebrate Tcfs can replace one or more vertebrate Tcf. These Tcf subtype

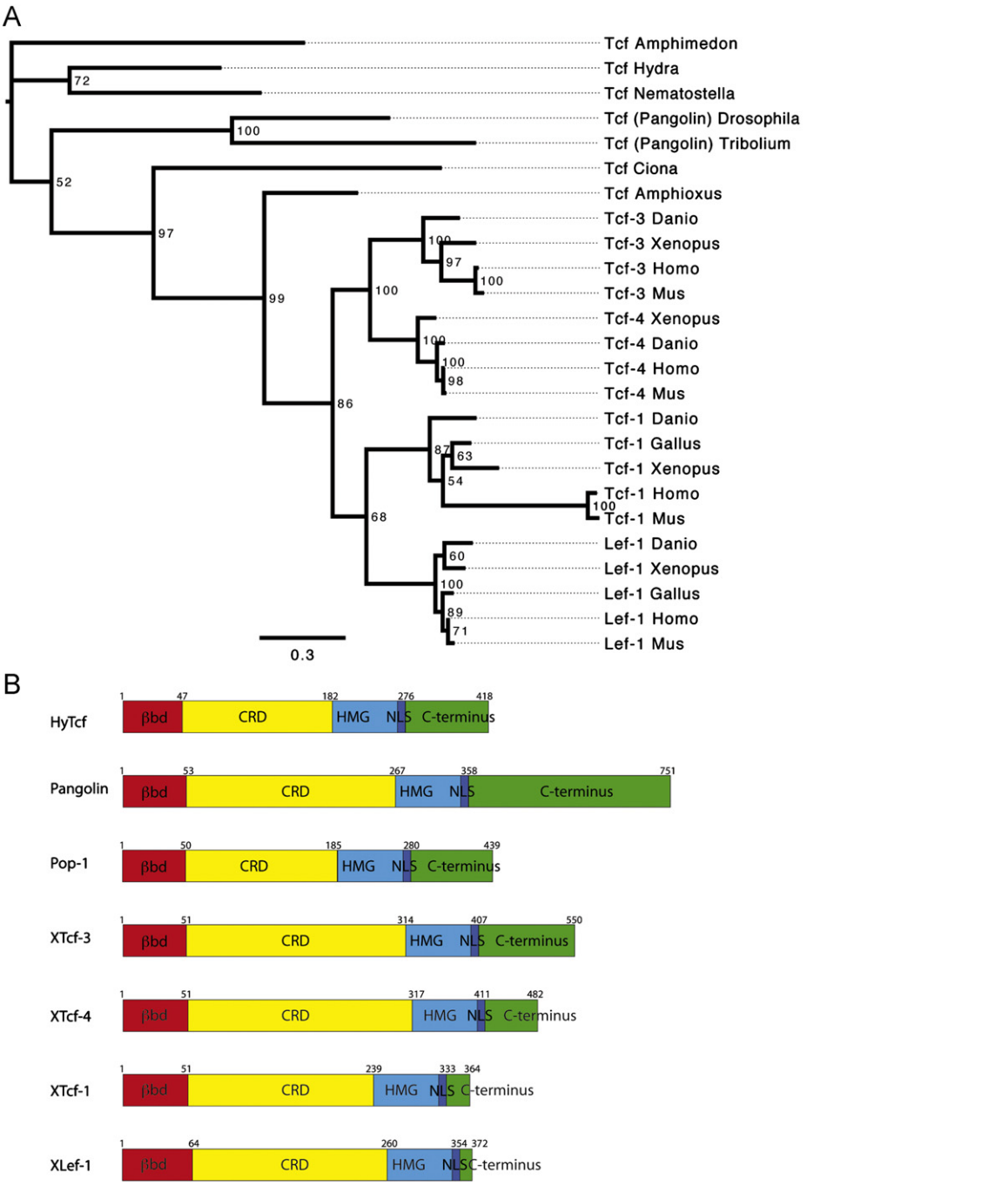

Fig. 1. Phylogenetic tree of Lef/Tcf transcription factors: (A) Maximum-likelihood tree of Lymphoid enhancer factor/T-cell factor (Lef/Tcf) proteins of invertebrates and vertebrates based on their complete amino acid sequences. Amphimedon Tcf is used as an outgroup. The numbers at the nodes are bootstrap support percentages. The scale bar indicates evolutionary distance in amino acid substitutions per position. (B) Schematic drawing illustrating domain positioning in Lef/Tcf transcription factors. Numbers are according to amino acid position.

specific assays were for Tcf-3, the expression of cold inducible RNA binding protein (cirp), for Tcf-4, the expression of engrailed 2 (en2), and for Tcf-1, the expression of Tcf-4 and subsequently en-2.

As shown previously (Gradl et al., 2002), ventral overexpression of XLef-1, but not XTcf-3 and XTcf-4, results in a duplication of the primary body axis. Here we can show that XTcf-1 acts similar to Lef-1 as a potent axis inducer (Fig. 2A, B). Thus, Tcf-1 and Lef-1 exhibit a function in the axis induction assay, which is lacking in Tcf-3 and -4. To our surprise, none of the invertebrate Tcfs ever induced a secondary axis (Fig. 2A, B). Instead, all invertebrate Tcfs inhibit secondary axis formation when co-injected together with XTcf-1 (data not shown). Thus, in the Xenopus axis induction assay, the invertebrate Tcfs show a functional similarity to Tcf-3 and some Tcf-4 splice variants, which all act as a repressor in the axis induction assay.

Consistent with this finding the activation of the SuperTOP-Flash promoter and the siamois promoter by invertebrate Tcfs was weak compared to the strong activation by XTcf-1 (Fig. 2C, D). This might implicate that transcriptional activation is a function that evolved in Tcf-1 and Lef-1 subfamilies only after the separation of Tcf-3 and Tcf-4. It also suggests that the ability to induce a secondary axis is a novel function of vertebrate Tcf-1 and Lef-1. If so, this novel function developed independently in Lef-1 and Tcf-1 because strong Wnt pathway activation by XTcf-1

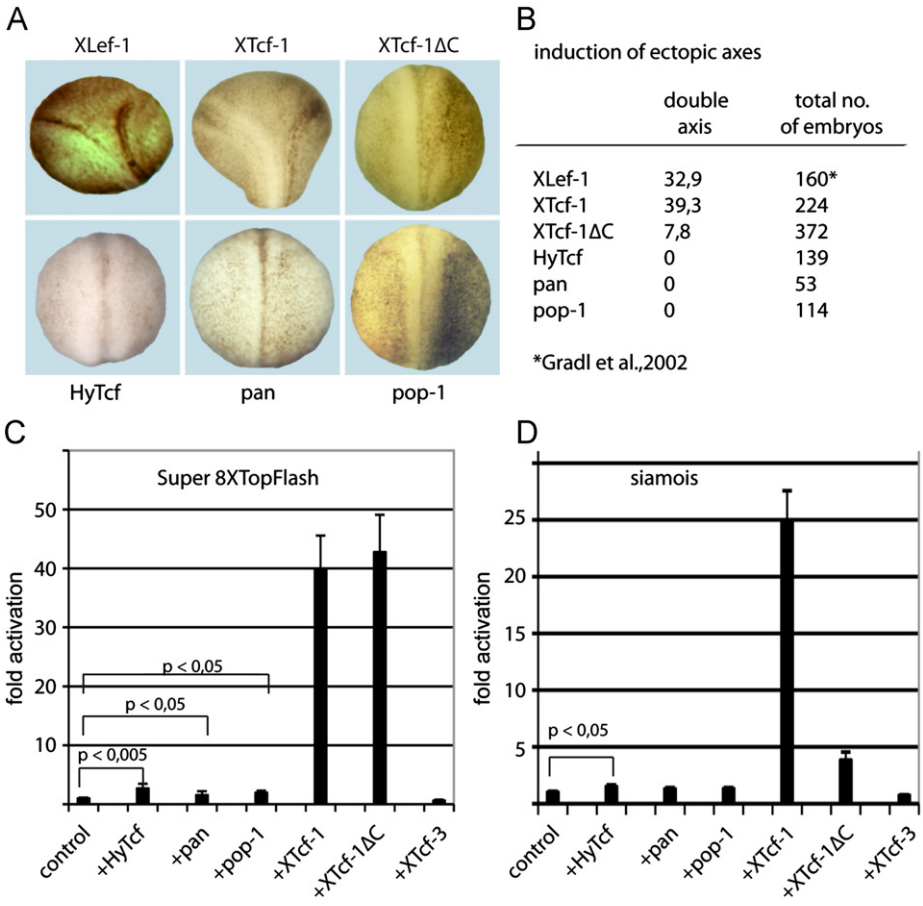

Fig. 2. Invertebrate Tcfs do not induce a secondary axis (A, B) and do not activate Wnt responsive promoters (C, D): (A) 500 pg mRNA of the indicated Lef/Tcf transcription factors was injected into both ventral blastomeres of *Xenopus* four-cell stage embryos, cultivated until neurula stages and scored for axis duplication, (B) HEK293 cells co-transfected with different Tcf-constructs and Super8xTopFlash and (C)*Xenopus siamois* promoter (D) was used to analyze whether invertebrate Tcfs activate the Wnt responsive promoters in a similar manner as vertebrate Tcfs. Given are mean values and standard errors of at least 8 transfections and *p*-values according to Students *t*-test.

requires a domain, the CRARF-containing C-terminus, which does not exist in Lef-1. Deletion of this C-terminus (XTcf-1ΔC) results in a construct, which is inactive in the secondary axis assay (Fig. 2A, B) and in the *siamois* promoter assay (Fig.2D), but still activates the SuperTOPFlash promoter in a similar manner as that of wild-type XTcf-1 (Fig. 2C). However, an alternative and from an evolutionary perspective a more probable explanation is that Tcf-1 and Lef-1 evolved new regulatory features that are required in the specific context of Tcf activation of vertebrates. This scenario would fit with the strong axis inducing capacity of the canonical Wnt pathway already present in cnidarians (Nakamura et al., 2011). Furthermore, only such a scenario can explain why some human Tcf-4 isoforms can induce a secondary axis (not shown).

Next, we knocked down *Xenopus* Tcf-1 by injecting antisense morpholino oligonucleotides and asked whether invertebrate Tcfs can substitute for XTcf-1 depletion. We have recently shown that XTcf-1 regulates the expression of XTcf-4 in the midbrain and that XTcf-4 is essential for driving Xen2 expression at the isthmus organizer (Kunz et al., 2004; Koenig et al., 2010). Consistently, Xen2 expression in XTcf-1 depleted embryos was reduced and partially restored by co-injected XTcf-4 (Fig. 3A, B). Two invertebrate Tcfs, i.e. HyTcf and Pangolin, were able to substitute for loss of XTcf-1 in a similar manner to that of XTcf-4 and partially restored Xen2 expression in XTcf-1 morphants (Fig. 3A, B). Only in Pop-1 co-injected embryos, Xen2 expression was not restored (Fig. 3A, B). Thus, Pop-1 can substitute neither for a loss of XTcf-1 nor for a loss of XTcf-4.

To decipher whether HyTcf and Pangolin can replace exclusively XTcf-1 or XTcf-4, or both, we analyzed in XTcf-1 depleted embryos the expression of XTcf-4 (Fig. 3C, D) and in XTcf-4 depleted embryos the expression of Xen2 (Fig. 3 E, F). Our results clearly demonstrate that Pangolin can substitute for the loss of XTcf-4 and XTcf-1. Both XTcf-4 expression in XTcf-1 morphants and Xen2 expression in XTcf-4 morphants were partially restored by co-injected Pangolin. HyTcf, instead, can replace only XTcf-4, but not XTcf-1. Co-injected HyTcf in XTcf-4 morphants restored Xen2 expression, while XTcf-4 expression in XTcf-1 morphants was not restored. As expected, co-injected Pop-1 was unable to restore marker gene expression following XTcf-1 and XTcf-4 depletion.

We recently identified the cold inducible RNA binding protein Xcirp as XTcf-3 subtype specific Lef/Tcf target (van Venrooy et al., 2008). We used this assay to test Tcf-3 function. In XTcf-3 morphants almost 80% of the injected embryos showed a severe reduction of Xcirp expression. Co-injection of Pangolin and Pop-1, but not HyTcf, restored Xcirp expression in XTcf-3 depleted embryos in a similar manner as that XTcf-3 (Fig. 4A, B). Considering XTcf-3 as a repressor and keeping in mind that high nuclear Pop-1 hinders Wnt driven transcription in *Caenorhabditis*, this rescue experiment in *Xenopus* might be simply explained by general activator/repressor functions.

Repressing domains of XTcf-3 dominate activating domains of XTcf-1

It has been discussed for a long time that the long C-terminus of XTcf-3, especially the recruitment of Ctbp, might be a key for XTcf-3 specific function. To test this, we created.

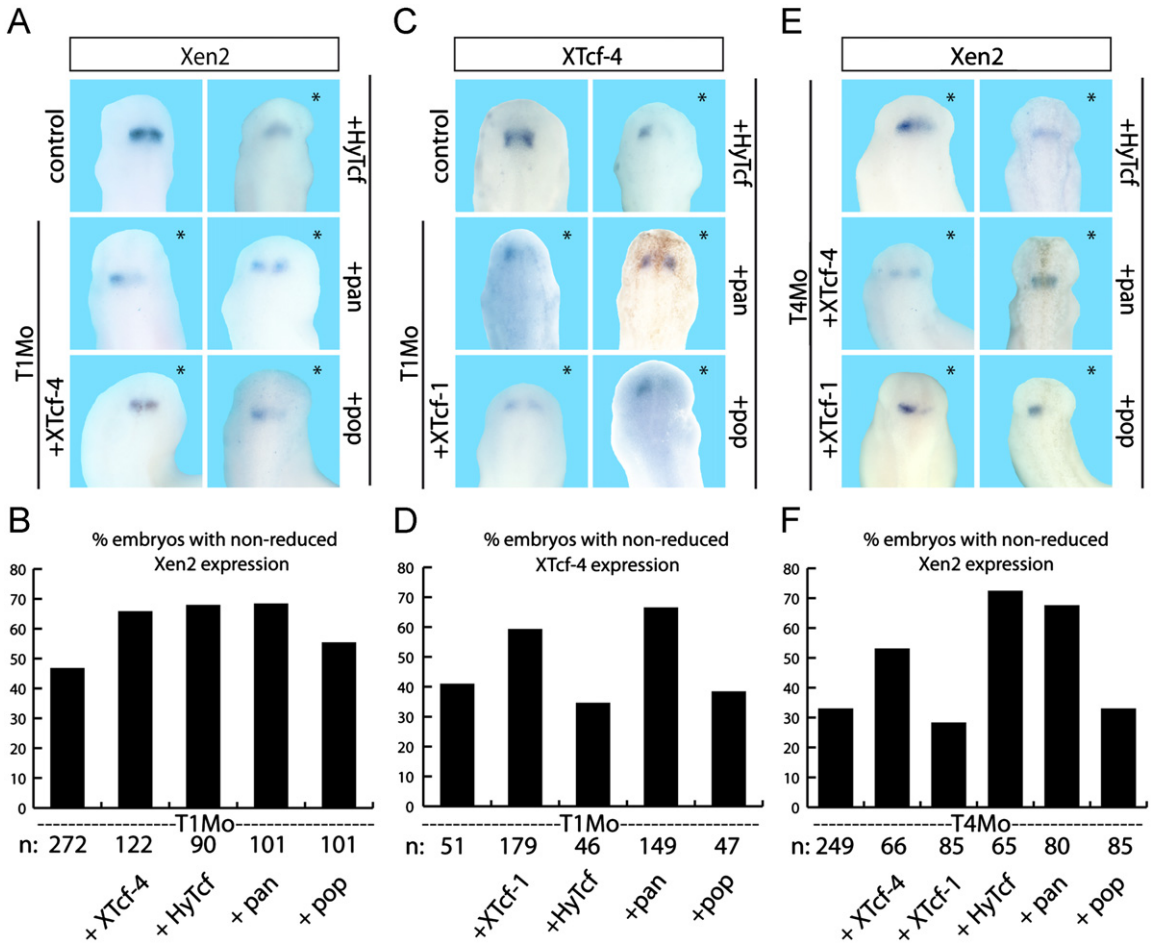

Fig. 3. Pangolin can replace XTcf-1 and XTcf-4, HyTcf can replace XTcf-4. (A) Expression of Xengrailed 2 (Xen2) in the isthmus organizer is reduced in XTcf-1 depleted embryos (T1Mo) and partially restored following co-expression of XTcf-4 (T1Mo+XTcf4), HydraTcf (T1Mo+HyTcf) and Pangolin (T1Mo+pan), but not after co-injection of Pop-1 (T1Mo+pop). 4 pMol XTcf-1 specific morpholino antisense oligonucleotide (T1Mo) was co-injected with 500 pg cDNA of the indicated Tcfs in the animal hemisphere of one blastomere in two-cell stage embryos. The asterisks mark the injected site. (B) Quantification of the *in situ* hybridization results shown in (A); *N*=number of analyzed embryos. (C) Expression of XTcf-4 in the midbrain is reduced in XTcf-1 depleted embryos and partially restored following co-expression of Pangolin, but not after co-injection of HydraTcf and pop-1. (D) Quantification of the *in situ* hybridization results shown in (C). (E) Expression of Xengrailed 2 (Xen2) in the isthmus organizer is reduced in XTcf-4 depleted embryos (T4Mo) and partially restored following co-expression of XTcf-4 (T4Mo+XTcf4), HydraTcf (T4Mo+HyTcf) and Pangolin (T4Mo+pan), but not after co-injection of Pop-1 (T4Mo+pop). 10 pMol XTcf-4 specific morpholino antisense oligonucleotide (T4Mo) was co-injected with 500 pg cDNA of the indicated Tcfs in the animal hemisphere of one blastomere in two-cell stage embryos. Quantification of the *in situ* hybridization results.

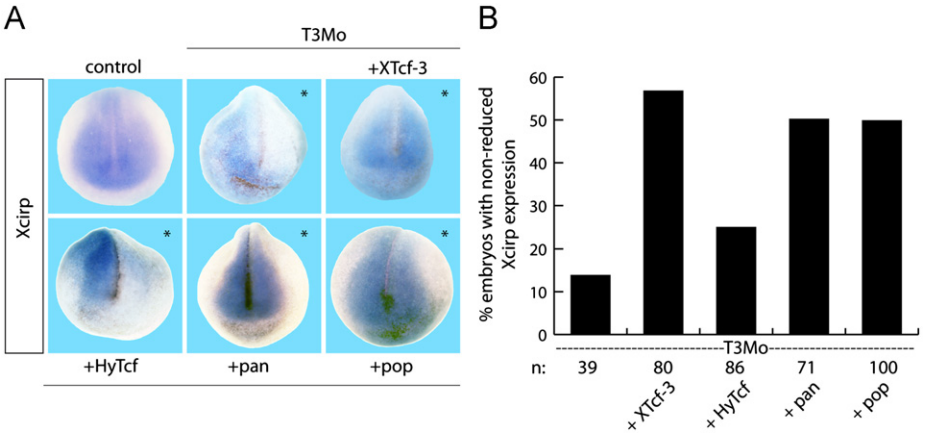

Fig. 4. Pangolin and Pop-1 can replace XTcf-3. (A) Expression of cold inducible RNA binding protein (Xcirp) in the neural plate is reduced in XTcf-3 depleted embryos (T3Mo) and partially restored following co-expression of Pop-1 (T3Mo+pop) and Pangolin (T3Mo+pan), but not after co-injection of HydraTcf (T3Mo+HyTcf). 2 pmol XTcf-3 specific morpholino antisense oligonucleotide (T3Mo) were co-injected with 1000 pg mRNA of the indicated Tcfs in the animal hemisphere of one blastomere in two-cell stage embryos. The asterisks mark the injected site. (B) Quantification of the *in situ* hybridization results shown in (A); N=number of analyzed embryos.

XTcf-1/XTcf-3 chimera and exchanged the C-termini of XTcf-3 and XTcf-1. We fused the long Ctbp-motif containing XTcf-3 C-terminus directly adjacent to the HMG box of XTcf-1 (XTcf-1/3) and the CRARF-containing XTcf-1 C-terminus directly adjacent to the HMG box of XTcf-3 (XTcf-3/1, Fig. 5A). Both chimeras failed to induce a secondary axis (supplementary Fig. 3A), although in reporter gene assays in HEK293 cells the XTcf-1/3 construct was a potent activator of the SuperTOPFlash and *siamois* promoters (supplementary Fig. 3B, C). Thus both the CRARF-containing C-terminus and the CRD of XTcf-1 are necessary, but none of them is sufficient for secondary axis formation. Again, *siamois* promoter reporter gene assays in transient transfectants reflect the *in vivo* function better than SuperTOPFlash assays.

Furthermore, any repressing motif of XTcf-3 (i.e. the CRD or C-terminus) seems to dominate the activating domains in XTcf-1. If this idea holds true, one would expect that both chimeras the XTcf-3/1 construct, and the XTcf-1/3 construct compensate the loss of the repressor XTcf-3 but not the loss of the activator XTcf-1. To test this, we co-injected the chimeras together with the XTcf-3 specific morpholino and together with the XTcf-1 specific morpholino. Indeed, both chimera partially restored Xcirp expression in XTcf-3 morphants (Fig. 5B, C), but they did not restore XTcf-4 expression in XTcf-1 morphants (Fig. 5D, E). So similar to the axis induction assay, the Tcf subtype specific assays revealed that repressing domains in XTcf-3 (CRD and C-terminus) dominate activating domains in XTcf-1. Repression via the long

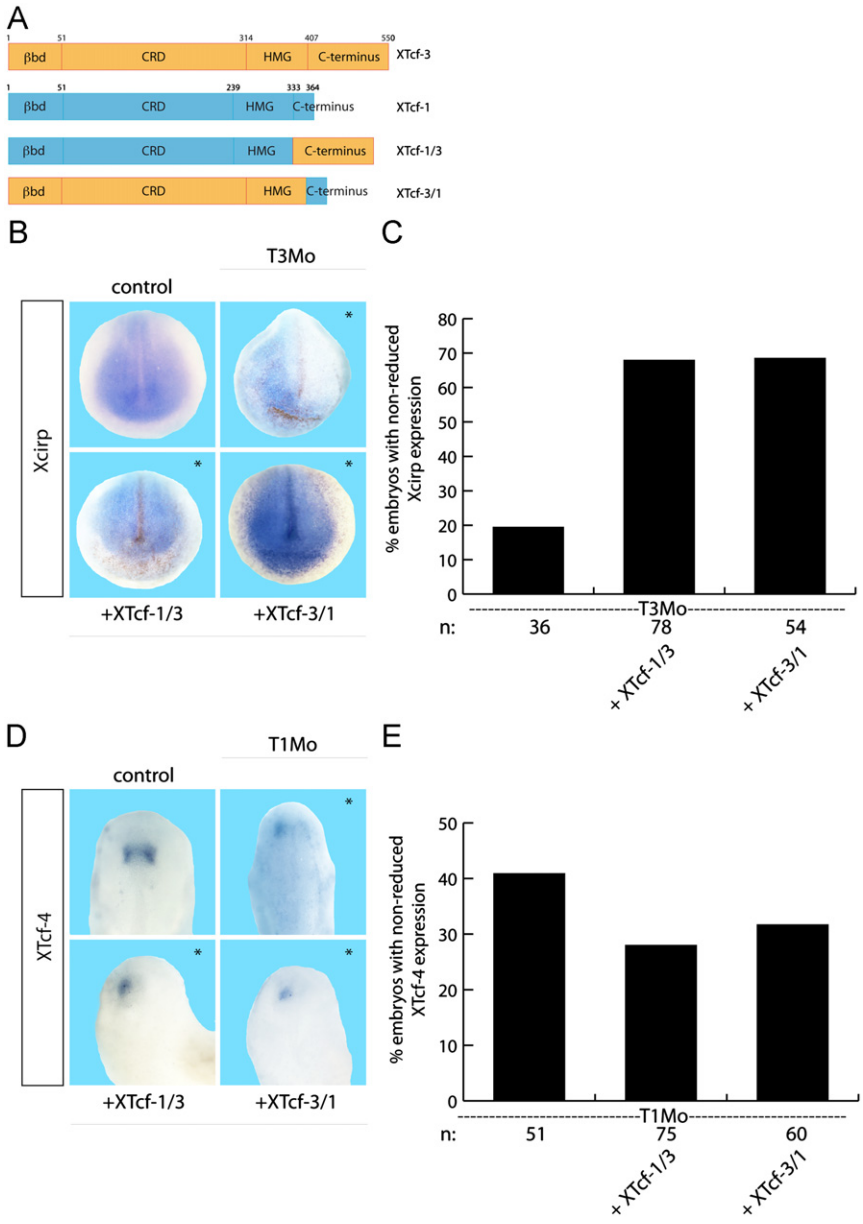

Fig. 5. Repressing motifs in XTcf-3 dominate activating motifs in XTcf-1. (A) We exchanged the C-termini of XTcf-3 (light gray) and XTcf-1 (dark gray) and fused the long Ctbp-motif containing XTcf-3 C-terminus directly adjacent to the HMG-box of XTcf-1 (XTcf-1/3) and the CRARF-containing XTcf-1 C-terminus directly adjacent to the HMG-box of XTcf-3 (XTcf-3/1). The numbers indicate the amino acids in the corresponding proteins. (B) Expression of cirp in the neural plate is reduced in XTcf-3 depleted embryos (T3Mo) and partially restored following co-expression of the XTcf-1 construct with the XTcf-3 C-terminus (T3Mo+XTcf-1/3) and the XTcf-3 construct with the XTcf-1 C-terminus (T3Mo+XTcf-3/1. (C) Quantification of the *in situ* hybridization results shown in (B); *N*=number of analyzed embryos. (D) Expression of XTcf-4 in the midbrain is reduced in XTcf-1 depleted embryos (T1Mo) and not restored following co-expression of XTcf-1 construct with the XTcf-3 C-terminus (T3Mo+XTcf-1/3) and the XTcf-3 construct with the XTcf-1 C-terminus (T3Mo+XTcf-3/1). The asterisks mark the injected site. (E) Quantification of the *in situ* hybridization results shown in (D); *N*=number of analyzed embryos.

C-terminus of XTcf-3 appears to be redundant with repression via the CRD of XTcf-3. Similar to what we have shown (Gradl et al., 2002), we also found in this study that the C-terminus of XTcf-3, and thus Ctbp binding, is of minor relevance for XTcf-3 specific functions. Even if the PSLVS motif-containing C-terminus of XTcf-3 is replaced by the partial C-clamp containing C-terminus of XTcf-1, target gene promoters are not activated and the resulting chimera still can replace XTcf-3 in the embryo. However, in this case we can show that the C-terminus of XTcf-3 is sufficient to assign Tcf-3 specific repressor functions when fused to the core of XTcf-1.

CRD is responsible for Tcf subtype specific functions

To determine which domains of a Tcf provides the transcription factor with its specific functions, we swapped the β-Catenin binding site, the CRD, the HMG box and the C-terminus of the functionally completely unrelated XTcf-1 and HyTcf (Fig. 6A) and tested first in gain of function experiments whether these chimeras behave XTcf-1-like or HyTcf-like. Surprisingly, none of the XTcf-1/HyTcf chimera induced a secondary axis (supplementary Fig. 4). Even swapping only the β-Catenin binding site, a construct that is composed of the βbs of HyTcf, the CRD, HMG-box and C-terminus of XTcf-1, resulted in a chimera (H111) unable to induce a secondary axis although this construct activated the SuperTOPFlash in a similar manner as that of XTcf-1 (supplementary Fig. 4). Thus, for the novel Lef/Tcf function, the induction of a secondary axis, all domains of XTcf-1 are relevant.

These findings are consistent with the activation of the *siamois* reporter in HEK293 cells (supplementary Fig. 4). Again, all chimeric constructs failed to robustly activate this wnt target promoter. However, the constructs containing the CRD of XTcf-1 activated the *siamois* promoter better than HyTcf or chimera with the CRD of HyTcf. This argues for an important role of the CRD in regulating Tcf-1 target genes and Tcf subtype specific functions. The importance of the CRD is seen even better in the Super-TOPFlash assay (supplementary Fig. 4, where all constructs with the CRD of XTcf-1 activated the promoter to a similar extent as did XTcf-1, while all constructs with the CRD of HyTcf were poor activators, similar to HyTcf.

Interestingly, there is no correlation between target gene activation or axis induction and the interaction with β-Catenin, because in a GST pulldown assay both HyTcf and XTcf-1 bind to recombinant vertebrate β-Catenin (supplementary Fig. 5). Considering the axis induction assay with our XTcf-1/HyTcf and XTcf-1/XTcf-3 chimera we favor the idea that for secondary axis induction in vertebrates, a complete Tcf-1 version is necessary because only wildtype XTcf-1 acts as a strong activator. Any domain of an unrelated Tcf compromises its activity. Fusion of the C-terminus of XTcf-3 and of HyTcf to the core of XTcf-1 resulted in constructs unable to induce an ectopic body axis.

This cannot be explained solely by the CRARF domain in the C-terminus of XTcf-1 for the following reasons: (i) XTcf-1 contains only an incomplete C-clamp, similar to the hTcf-1 short isoform, which in mammalian cell culture systems was shown to be a poor activator (Weise et al., 2010), (ii) the C-terminus of HyTcf that contains a CRARF motif which, based on its sequence, should form a complete C-clamp, suppresses secondary axis formation when fused to the core of XTcf-1 and (iii) Fusion of the C-terminus of XTcf-1 to the core of XTcf-3 did not result in a construct able to induce ectopic axes and activate Wnt responsive promoters. Because even swapping of the β-Catenin binding site abolished axis formation, we think that the ability of XTcf-1 to induce a secondary axis relies on overall structural properties of this particular Tcf, including the very N-terminus and the C-terminus.

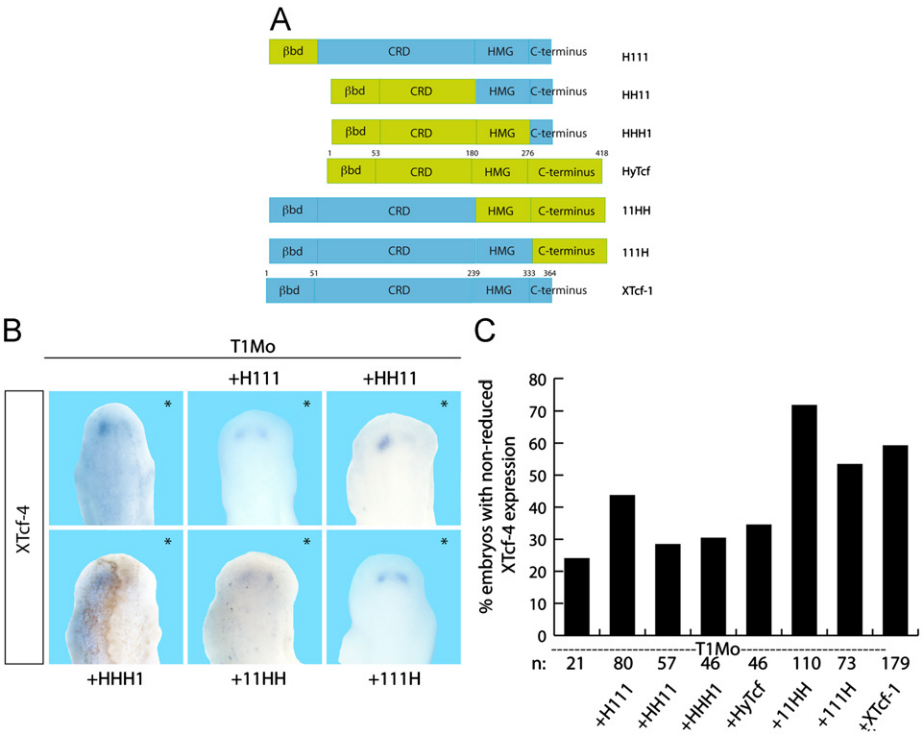

Fig. 6. Context dependent regulatory domain is essential for XTcf-1 specific function. Chimera consists of different parts of HyTcf (light gray) and XTcf-1 (dark gray). The numbers indicate the amino acids in the corresponding proteins. Swapping of the β-Catenin binding site (βbd) and context dependent regulatory domain (CRD) of XTcf-1 and HyTcf results in the chimeras HH11 and 11HH. Exchanging the βbs of XTcf-1 by the βbs of HyTcf results in the chimera H1111, swapping the C-terminus directly adjacent to the HMG-box results in the chimera HHH1 and 111H. (B) Expression of XTcf-4 in the midbrain is reduced in XTcf-1 depleted embryos (T1Mo) and partially restored following co-expression of those chimeras that contain the CRD of XTcf-1 (T1Mo+11HH, 111H and H111), but not after co-injection of chimera with the CRD of HydraTcf (T1Mo+HH11 and HHH1). The asterisks mark the injected site. (C) Quantification of the *in situ* hybridization results shown in (B); *N*=number of analyzed embryos.

Next we used the chimeric constructs to test whether they are able to restore XTcf-4 expression in XTcf-1 morphants. These experiments showed that those chimeras that contain the CRD of XTcf-1 do partially rescue the XTcf-1 morphant phenotype; the chimera with the CRD of HyTcf has no effect (Fig. 6 B, C). Thus, for regulating XTcf-4 expression in the developing embryo, the CRD of XTcf-1, but not the β-Catenin binding domain, the HMG box and the C-terminus, is necessary. The latter domains may be provided by the distantly related HyTcf.

Taken together the capacity to induce a secondary axis is a novel and vertebrate Lef/Tcf subtype specific function, which involves changes in several domains including β-binding site, CRD and C-terminus. Subfunctionalization, instead, appears to rely mostly on the CRD as a modulatory region, which provides at least XTcf-1 and XTcf-3 with their subtype specificity.

Evolutionary considerations

In our functional analysis using different XTcf morphants we found that only Pangolin from insects could replace all, Tcf-1, Tcf-3, and Tcf-4 (Figs. 3 and 4). By comparison, only XTcf-3 morphants were replaced by Pop-1, and only XTcf-4 morphants by HyTcf (Table 1).

In an evolutionary context these data cannot be easily reconciled. Tcfs from *Hydra* and *Caenorhabditis elegans* – in the tree of life both species are more basal than *Drosophila* – failed to rescue all *Xenopus* Tcfs, but instead restored distinct family members. This suggests that the common ancestor of protostomes and deuterostomes shared a Tcf transcription factor with similar functions in canonical Wnt signaling. The fact that even the *Hydra* Tcf can replace some of the vertebrate Tcfs further implicates that this property was even a feature already present in the last common ancestor of all eumetazoan species. A divergence of *Hydra* and Caenorhabditis within the cnidarian and ecdysozoan clade might explain why Hydra and Caenorhabditis Tcfs cannot substitute all vertebrate Tcfs.

The fact that Pangolin replaced all *Xenopus* Tcfs tested indicates that at least the common ancestor of insects and vertebrates had an ancient function, probably also in axis formation. In the short-germ insect *Tribolium castaneum*, *Wnt* genes and Pangolin are required for the segmentation process of the embryo (Bolognesi et al., 2008), which is in line with this hypothesis. If so, it is parsimonious to assume that also the common bilaterian ancestor shared a Tcf transcription factor with functions in canonical Wnt signaling and axis induction. The fact that only Tcf-1 and Lef-1 (but none of the invertebrate Tcfs) induced a secondary axis following ventral injection might be explained by an evolutionary scenario where Tcf-1 and Lef-1 used existing modules present in the common ancestor.

Considering the function of invertebrate Tcfs in their natural context, it is well established, that Pangolin acts as an activator and a repressor of downstream target genes (Blauwkamp et al., 2008; Schweizer et al., 2003; Hoffmans et al., 2005). We therefore conclude that activating and repressing properties of vertebrate Lef/Tcfs were already present in the Tcf of the common bilaterian ancestor. It should be emphasized that Hydra and Caenorhabditis Tcfs cannot substitute all vertebrate Tcfs. This, however, might be related to the divergence of *Hydra* and Caenorhabditis within the cnidarian and ecdysozoan clades. The Caenorhabditis Tcf Pop-1 was described as an activator and repressor of Wnt target genes (Korswagen et al., 2000; Calvo et al., 2001; Owraghi et al., 2010). In line with the high divergence of nematode worms is the fact that 4 different β-Catenin homologs have been described for Caenorhabditis, i.e. bar-1, wrm-1, sys-1 and hmp-2 (Natarajan et al., 2001; Takeshita and Sawa, 2005; Liu et al., 2008) that may define the outcome of Pop-1 target gene regulation.

On the molecular level we presume that the failure of Pop-1 to restore XTcf-3 depletion, but not XTcf-1 and XTcf-4 depletion, is due to differential phosphorylation. It has been recently shown, that Tcf-3, Tcf-4 and Lef-1 but not Tcf-1 are regulated by Wnt dependent phosphorylation through homeodomain interacting protein kinase 2 (HIPK2) at distinct serine residues in the CRD (Hikasa et al., 2010, Hikasa and Sokol, 2011). Interestingly, some of the relevant phosphorylation sites, including P2 and P4, are conserved in some invertebrate Tcfs. Thus, HIPK dependent repression of Tcf-1 got lost after the second gene duplication and might be one explanation for strong target gene activation by XTcf-1. Very recently, Robertson et al. (2011) showed that hTcf-4 but not mLef-1 and XTcf-3 can partially replace Pop-1 in *Caenorhabditis*, indicating that the common ancestor of Pop-1 and vertebrate Tcfs united features which were subdivided in the vertebrate lineage on different transcription factors. In *Hydra*, it remains unclear whether endogenous HyTcf activates or represses Wnt target genes in Cnidaria, or whether it can switch in a Wnt dependent manner from an activator to a repressor state.

In summary our data provide evidence for a non-redundant Tcf subtype specific function that can be explained by subfunctionalization. We presume that the functional properties of the common ancestor Tcf characterized by context dependent activation and repression were subdivided on Tcf-1, Lef-1, Tcf-3 and Tcf-4. This subfunctionalization seems to affect mostly the CRD as a modulatory region, which provides at least XTcf-1 and XTcf-3 with their subtype specificity. An open question that deserves further studies is the fact that function of secondary axis induction in vertebrates must have involved changes in several domains, including β-Catenin binding site, CRD and C-terminus. Future study will reveal whether subtype specific phosphorylation similar to what is known for XTcf-3 (Hikasa et al., 2010; Hikasa and Sokol, 2011) and/or subtype specific recruitment of co-factors accounts for the Tcf subtype specific characteristics.

Material and methods

Phylogeny

Amino acid sequences of *Amphimedon*, *Hydra*, *Nematostella*, *Tribolium*, *Drosophila*, *Ciona*, *Amphioxus*, and vertebrate Lef/Tcf proteins

Table 1
Summarization of overexpression and rescue experiments.

	Secondary axes (%)	Promoter activation		Rescue of XTcf morpholino injections		
		Siamois	TOPFlash	XTcf-1 morphants	XTcf-4 morphants	XTcf-3 morphants
Pan	0	x13	x 1,6	Yes	yes	Yes
Pop-1	0	x13	x 2,0	No	No	Yes
HyTcf	0	x15	x27	No	Yes	No
XTcf-1	39,3	x248	x400 (CRD)	Yes (CRD)	No	No
XTcf-3	0	x07	x06	No	No	Yes (CRD/C-terminus)
XTcf-4	0	x15 to x22 (ref. 17)	x10 (not shown)	Yes (for en2)[a]	Yes	No

[a] Based on Koenig et al. (2010) we suggest, that this is an indirect effect.

were aligned with ClustalW (Larkin et al., 2007). Proteins can be identified as follows: Tcf amphimedon (*Amphimedon queenslandica*, AD016566); Tcf amphioxus (*Branchiostoma floridae*, AAZ77711); Tcf ciona (*Ciona savignyi*, BAB68354); Tcf hydra (*Hydra magnipapillata*, AAG13664); Tcf nematostella *(Nematostella vectensis*, ABF55257); Tcf (Pangolin) drosophila (*Drosophila melanogaster*, P91943); Tcf (Pangolin) tribolium (*Tribolium castaneum*, NP_001034990); Tcf-1 Gallus (*Gallus gallus*, gi|21666820|gb|AAM73851.1|AF454504_1HMG); Tcf-1 zebrafish (*Danio rerio*, NP_001012389); Lef-1 Gallus (*Gallus gallus*, gi|45384346|ref|NP_990344.1); Lef-1 zebrafish (*Danio rerio*, NP_571501); Tcf-3 zebrafish (*Danio rerio*, Q9YHE8); Tcf-4 zebrafish (*Danio rerio,* NP_571334); Tcf-1 human (*Homo sapiens*, AAH48769); Lef-1 human (*Homo sapiens*, NP_057353); Tcf-3 human (*Homo sapiens*, NP_112573); Tcf-4 human (*Homo sapiens*, NP_110383); Tcf-1 mouse (*Mus musculus*, EDL33620); Lef-1 mouse (*Mus musculus*, NP_034833); Tcf-3 mouse (*Mus musculus*, CAA11070); Tcf-4 mouse (*Mus musculus,* NP_001136394); Tcf-1 xenopus (*Xenopus tropicalis*, AAO23662); Lef-1 xenopus (*Xenopus laevis*, AAK58834); Tcf-3 xenopus (*Xenopus laevis,* AAK58835); and Tcf-4 xenopus (*Xenopus laevis*, NP_001083866).

Phylogenetic trees were reconstructed using maximum likelihood (ML) and Bayesian methodsand the WAG model (Whelan and Goldman, 2001) assuming rate heterogeneity with 4 discrete Gamma rate categories (Yang, 1993). Missing parameters were estimated from the data and option set to default settings if not otherwise stated. Maximum likelihood trees (PhyML) were constructed according to Guindon and Gascuel (2003) and by computing 100–200 bootstrap trees. Neighbor-joining trees (Jukes–Cantor) were tested with 50,000 bootstrap replicates with *mphimedon* as an outgroup.

Bayesian trees were computed using MrBayes (Ronquist and Huelsenbeck, 2003) performing four runs with two chains running for 1.1 mio generations each. Every 200th tree was sampled from the cold chains after a burn-in of 500.

Plasmids and constructs

The open reading frames of Pangolin (Acc. No. M_166718) and HyTcf (Acc. No. AF271696.1) were inserted into XhoI site of pCS2-myc. Pop1 in pK-Myc-C3 was kindly provided by Masako Asahina. XTcf-1 (Acc. No. AAO23663), XTcf-3 (Acc. No. AAK58835), XTcf-4 (Acc. No. AF287151) and XLef-1 (Acc. No. AAK58834) are as described (Koenig et al., 2010; Pukrop et al., 2001). The chimera were constructed by combining different Tcf domains *via* PCR and subcloned at XhoI site of pCS2-myc. Primers sequences are available upon request.

Probes for *in situ* hybridization are described elsewhere: XTcf-4 and Xen2 (Koenig et al., 2010), and Xcirp (van Venrooy et al., 2008).

The following antisense morpholino oligonucleotides (Gene Tools) were used: XTcf-1: 5′-CGGCGCTGTTCATTTGGGGCAT-3′; XTcf-3: 5′-CGCTGTTGAGCTGAGGCATGATGAG-3′; XTcf-4: 5′-CGCCATTCAACTGCGGCATCTCTGC-3′.

Embryo manipulation

Capped mRNAs were transcribed *in vitro* from linearized DNA templates using mMESSAGE mMACHINE (Ambion). Indicated amounts of mRNA, DNA and antisense morpholino oligonucleotides were co-injected with 4 pg dextran-FITC as a lineage tracer into the animal hemisphere of one blastomere of two-cell stage *Xenopus* embryos or into the marginal zone of both ventral blastomeres of four-cell stage embryos. The dextran-FITC staining allowed us to distinguish left hand site injected embryos from right hand site injected embryos at neurula stages. The embryos were staged according to Nieuwkoop and Faber, (1967) and kept and fixed as described in van Venrooy et al. (2008).

Whole-mount *in situ* hybridization was performed as described in Harland (1991). To visualize the localization of mRNA we used an anti-digoxygenin antibody conjugated with alkaline phosphatase following incubation with nitro-blue tetrazolium (NBT) and 5-bromo 4-chloro 3-indolyl phosphatase (BCIP). Digoxygenin labeled antisense probes for *in situ* hybridization were synthesized with a DIG RNA labeling kit (Roche).

GST pulldown assay and reporter gene assays

Bacterially expressed GST-tagged human β-Catenin, green fluorescent protein and DCOH were immobilized on Glutathion sepharose beads for 2 h at 4 °C in NOP buffer (10 mM Tris/Cl, pH 7.8; 150 mM NaCl, 1 mM $MgCl_2.6H_2O$, 0.75 mM $CaCl_2.2H_2O$, 2% Nonidet P40) and incubated with NOP-lysate of transfected human kidney epithelial cells (HEK293). After binding for 2 h at 4 °C the samples were washed 3 times with NOP buffer, boiled in SDS sample buffer for 5 min and subjected to 10% SDS PAGE. Proteins were transferred onto nitrocellulose, probed with anti-myc antibody 9E10 and revealed by chemiluminescence reaction (ECL, Amersham). Reporter gene assays in transfected HEK cells were as described Gradl et al., 2002; Koenig et al., 2010). In brief; semiconfluent HEK293 cells were transfected with 1 μg luciferase reporter construct, 0.7 μg CMV–β-Galactosidase for normalization and 3 μg of the indicated transcription factor, and cultivated for 48 h before measuring luziferase and *β*-Galactodsidase activity.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.05.012.

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